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miR-133b Inhibits Cell Growth, Migration, and Invasion by Targeting MMP9 in Non-Small Cell Lung Cancer

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Although increasing evidence indicates that the deregulation of microRNAs (miRNAs) contributes to tumorigenesis and invasion, little is known about the role of miR-133b in human non-small cell lung cancer (NSCLC). In the present study, we revealed that the introduction of miR-133b dramatically suppressed NSCLC cell growth, migration, and invasion in vitro. On the contrary, miR-133b inhibitors promoted cell growth, migration, and invasion in vitro. Further studies revealed that matrix metallopeptidase 9 (MMP9) is a direct target gene of miR-133b. Silencing MMP9 inhibited cell growth, migration, and invasion of NSCLC cells, which was consistent with the effect of miR-133b overexpression. In clinical specimens, reduced miR-133b was an unfavorable factor and negatively correlated with MMP9 expression. Our studies demonstrate that miR-133b inhibits cell growth, migration, and invasion by targeting MMP9 in NSCLC.

Key words: miR-133b; Matrix metallopeptidase 9 (MMP9); Non-small cell lung cancer (NSCLC); Cell growth; Migration and invasion

INTRODUCTION

Lung cancer is the most common cancer in the world, with a survival rate of 15%[1]. Eighty percent of these cases involved non-small cell lung cancer (NSCLC). Surgical resection is known to be the most effective treatment for NSCLC. However, due to the fact that most diagnoses were confirmed at an advanced stage because of its deep location and no specificity of symptoms in its early stage, only a few patients can be cured by surgical treatment. Therefore, there is an urgent need to search valuable factors for early diagnosis, prognosis prediction, and novel therapeutic strategies.

MicroRNAs (miRNAs) play an important role in the development, cellular differentiation, growth, cell cycle control, cell death, and metastasis[2] and have been involved in various human diseases, including cancer[3,4]. Therefore, researching the aberrant expression pattern and the roles of miRNAs in NSCLC will be helpful in understanding the mechanism of NSCLC carcinogenesis and developing new methods for NSCLC diagnosis and therapy.

miR-133b, which was initially considered to be a muscle-specific miRNA[5,6], has been reported to be deregulated in many kinds of cancer[7–10]. The downregulation of miR-133b in NSCLC has also been reported by several groups[7,11,12]. However, the tumor-suppressor role of miR-133b in NSCLC and its detailed mechanism are largely unknown.

Thus, this study was designed to investigate the function and possible molecular basis of miR-133b in the pathogenesis of NSCLC. Here we demonstrated that miR-133b inhibited cell growth, migration, and invasion through directly targeting matrix metallopeptidase 9 (MMP9) in NSCLC, which in turn contributed to the pathogenesis of NSCLC.

MATERIALS AND METHODS

Cell Culture, Sample Collection, and Ethics Statement

NSCLC H1299 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Inc., Pasching, Austria) and were incubated in a humidified chamber with 5% CO_2 at 37°C. Forty fresh NSCLC tissues and their corresponding distal paracarcinoma tissues (≥5 cm) were obtained from the Affiliated Hospital of Guangdong Medical College, Zhanjiang City, P.R. China, at the time of diagnosis before any therapy had been done. Clinical processes were approved by the ethics

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Table 1. Sequences of Primers Used in This Study

Primer	Sequence
miR-133b	
Sense	5′-TTTGGTCCCCTTCAACCAGC-3′
Antisense	–
U6	
Sense	5′-CTCGCTTCGGCAGCACA-3′
Antisense	–
MMP9	
Sense	5′-TTGGTCCACCTGGTTCAACT-3′
Antisense	5′-ACGACGTCTTCCAGTACCGA-3′
GAPDH	
Sense	5′-GGGTGTGAACCATGAGAAGT-3′
Antisense	5′-CAGTGATGGCATGGACTGTG-3′
MMP9 3′-UTR-wt	
Sense	5′-tcgagCTTTGGCAGTGCCATGTAAATCCCCACTGGGACCAACCCTGGGGAAGGAGCCAg-3′
Antisense	5′-gatccTGGCTCCTTCCCCAGGGTTGGTCCCAGTGGGGATTTACATGGCACTGCCAAAGc-3′
MMP9 3′-UTR-mut	
Sense	5′-tcgagCTTTGGCAGTGCCATGTAAATCCCCACTGCCTGGTTCCCTGGGGAAGGAGCCAg-3′
Antisense	5′-gatccGGCTCCTTCCCCAGGGAACCAGGCAGTGGGGATTTACATGGCACTGCCAAAGc-3′

committee of the Affiliated Hospital of Guangdong Medical University. Patients provided informed consents.

RNA Isolation, Reverse Transcription, and qRT-PCR

RNA was extracted from H1299 cells, 40 NSCLC tissues, and their corresponding distal paracarcinoma tissues using TRIzol (Takara, Shiga, Japan). For miR-133b qRT-PCR, RNA was transcribed into cDNA and amplified with specific sense primers (Table 1), and general antisense primer was supplied by the manufacturer using the miRNA PrimeScript® RT Enzyme Mix Kit according to the manufacturer's instructions (Ambion, Grand Island, NY, USA). For MMP9 qRT-PCR, RNA was transcribed into cDNA and amplified with specific sense/antisense primer (Table 1). The assays were performed in accordance with the manufacturer's instructions (Takara). The PCR for each gene was repeated three times. miRNA and mRNA expressions were normalized to U6 and GAPDH (Table 1), respectively, using the $2^{-\Delta\Delta}$Ct method[13].

Transient Transfection With miR-133b Mimics/Inhibitor or MMP9 siRNAs

miR-133b mimics and inhibitor and MMP9 siRNAs were designed and synthesized (Tables 2 and 3) (Ribobio, Guangzhou, P.R. China). Twenty-four hours prior to transfection, NSCLC H1299 cells were plated onto 6-well or 96-well plates (Nest Biotech, P.R. China) at 30%–50% confluence. miR-133b mimics, its inhibitors, or MMP9 siRNAs were then transfected at a working concentration of 100 nM using TurboFect™ siRNA Transfection Reagent (Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol. Cells were collected after 48–72 h for further experiments.

Colony Formation Assay

Cells were plated into six-well culture plates at a density of 200 cells/well. Each cell group had two wells. After incubation for 12 days at 37°C, cells were washed twice with PBS, formalin fixed, and stained with the Giemsa solution. The number of colonies containing ≥50 cells

Table 2. Sequences of miR-133b Mimics and Negative Control, miR-133b Inhibitor, and Inhibitor Negative Control

Gene	Sequence
miR-133b mimics	
Sense	5′-UUUGGUCCCCUUCAACCAGCUA-3′
Antisense	5′-UAGCUGGUUGAAGGGGACCAAA-3′
Negative control	
Sense	–
Antisense	–
miR-133b inhibitor	5′-UAGCUGGUUGAAGGGGACCAAA-3′
Inhibitor negative control	–

Table 3. Sequences of MMP9 siRNA

No.	Sequence
1	
Sense	5′-CCACCACAACAUCACCUAU dTdT-3′
Antisense	3′-dTdt GGUGGUGUUGUAGUGGAUA-5′
2	
Sense	5′-GCAUAAGGACGACGUGAAU dTdT-3′
Antisense	3′-dTdT CGUAUUCCUGCUGCACUUA-5′
3	
Sense	5′-GGAACCAGCUGUAUUUGUU dTdT-3′
Antisense	3′-dTdT CCUUGGUCGACAUAAACAA-5′

was counted under a microscope. The colony formation efficiency was calculated as (number of colonies/number of cells inoculated)×100%.

In Vitro Cell Migration and Invasion Assays

Twenty-four hours prior to transfection, NSCLC H1299 cells were plated onto a six-well plate (Nest Biotech) at 30%–50% confluence. miR-133b mimics or inhibitors were then transfected at a working concentration of 100 nM using TurboFect™ siRNA Transfection Reagent (Fermentas) according to the manufacturer's protocol. Cells were collected after 24 h for further experiments. Briefly, 1×10^5 cells were seeded onto a fibronectin-coated polycarbonate membrane insert in a Transwell apparatus (Corning, Corning, NY, USA). After the cells were incubated for 12 h, Giemsa-stained cells adhering to the lower surface were counted under a microscope in five predetermined fields (100×). For the cell invasion assay, the procedure was similar to that for the cell migration assay except that the Transwell membranes were precoated with 24 mg/ml Matrigel (R&D Systems, Minneapolis, MN, USA).

Western Blot Analysis

Cells were lysed in RIPA buffer (Beyotime, Shanghai, P.R. China), and protein concentration was determined using BCA assay (Beyotime). Total protein (30 μg) was

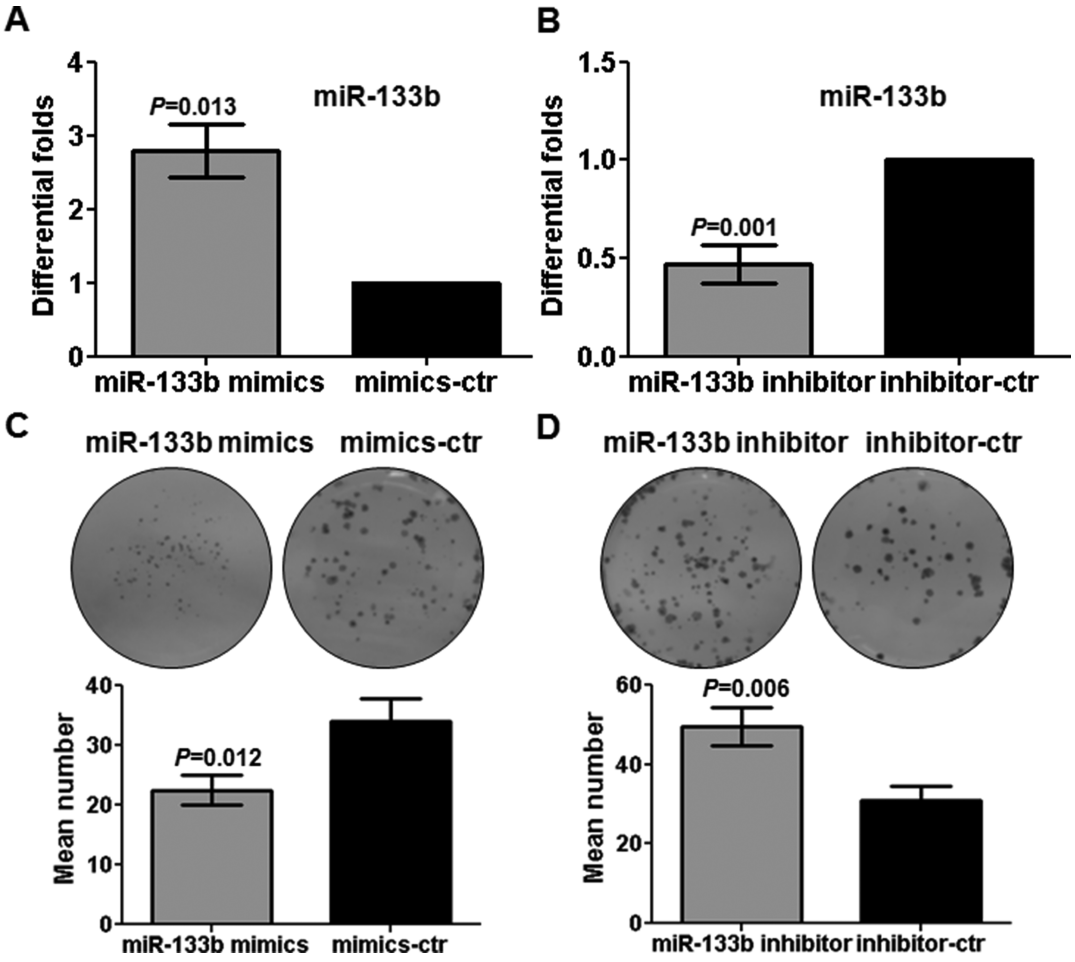

Figure 1. miR-133b suppressed cell growth of non-small cell lung cancer (NSCLC) H1299 cells. (A, B) miR-133b mimics or miR-133b inhibitor induced or reduced the expression of miR-133b in NSCLC H1299 cells. (C, D) miR-133b mimics or miR-133b inhibitor inhibited or elevated cell growth of NSCLC H1299 cells. Data were presented as mean±SD for three independent experiments.

resolved using a 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to polyvinylidene fluoride membranes (Invitrogen, Carlsbad, CA, USA). Membranes were blocked with 5% nonfat dry milk (for phosphorylation antibody, adding BSA) in Tris-buffered saline (pH 7.5), followed by immunoblotting overnight at 4°C with anti-MMP9 antibody (1:1,000; Abcam, Danvers, MA, USA) and anti-β-actin antibody (1:1,000; Proteintech, Chicago, IL, USA). HRP-conjugated anti-rabbit and anti-mouse IgG antibody was used as the secondary antibody (Zhongshan, Beijing, P.R. China). Signals were detected using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

miRNA Target Validation

MMP9 was predicted to be a directly regulated target of miR-133b by TargetScan. A 192-bp fragment of MMP9 3′-UTR amplified by PCR primers (Table 1) was cloned into pLUC vector [named wild type (wt)]. Site-directed mutagenesis of the miR-133b-binding site in MMP9 3′-UTR (Table 1) was performed using GeneTailor Site-Directed Mutagenesis System (Invitrogen) [named mutant (mut)]. For reporter assays, wt or mut vector was cotransfected into H1299 cells with miR-133b mimics or negative control. Luciferase activity was measured at 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

All data were analyzed for statistical significance using SPSS 13.0 software. Data are expressed as the mean±SD from at least three independent experiments. Comparisons between two groups were performed using Student's *t*-test. Associations between miR-133b and MMP9 were analyzed using Spearman's correlation coefficient. A value of $p<0.05$ was considered statistically significant.

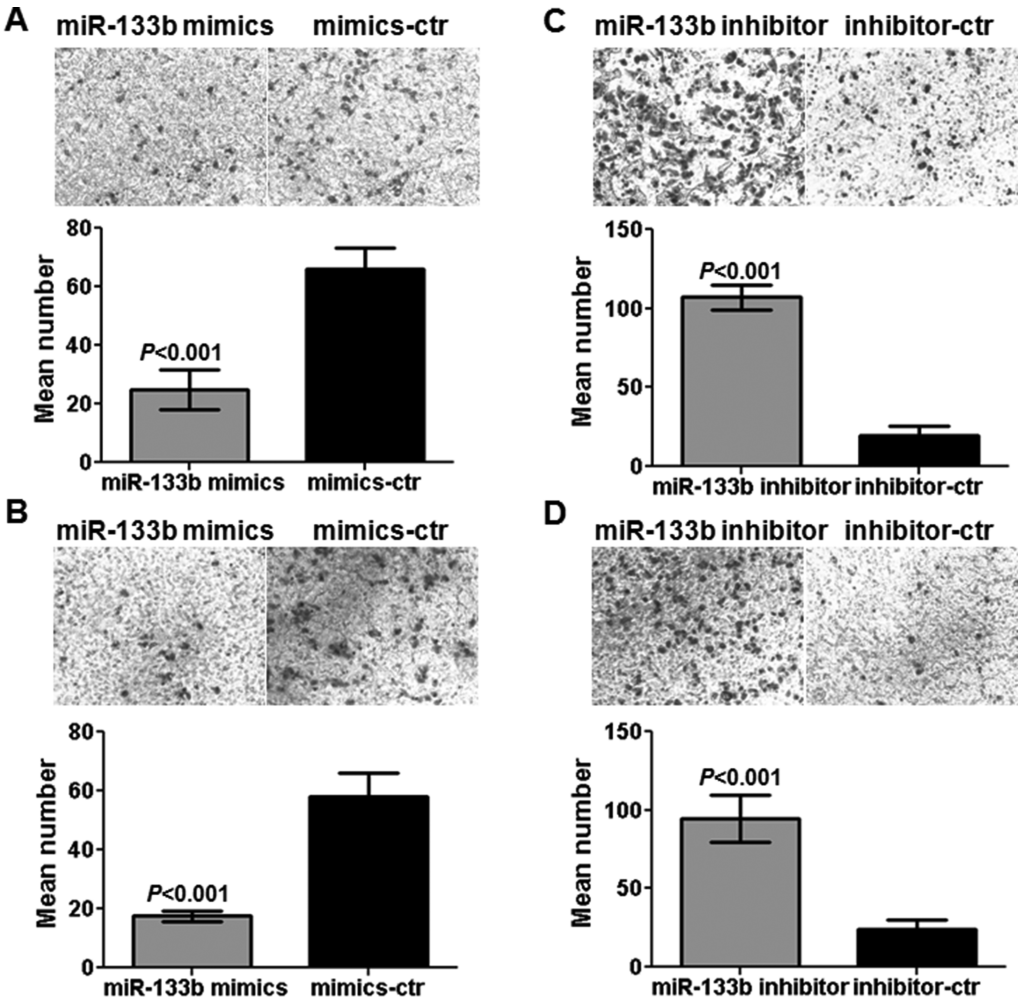

Figure 2. miR-133b inhibited cell migration and invasion of NSCLC H1299 cells. (A, B) miR-133b mimics reduced cell migration and invasion of NSCLC H1299 cells. (C, D) miR-133b inhibitor induced cell migration and invasion of NSCLC H1299 cells. Data were presented as mean±SD for three independent experiments.

RESULTS

miR-133b Suppresses H1299 Cell Growth In Vitro

To further explore its biological role in NPC, miR-133b mimics or inhibitors were, respectively, introduced into NSCLC H1299 cell lines to induce or reduce the expression of miR-133b (Fig. 1A and B). Compared with the negative controls, miR-133b mimics inhibited cell growth (Fig. 1C) by colony formation assay. Inversely, knocking down miR-133b by its specific inhibitors significantly restored cell proliferation (Fig. 1D).

miR-133b Inhibits H1299 Cell Migration and Invasion In Vitro

Compared with the negative controls, miR-133b mimics reduced cell migration and invasion in Transwell (Fig. 2A) and Boyden assays (Fig. 2B). Conversely, suppressing miR-133b by its specific inhibitors elevated cell migration (Fig. 2C) and invasion (Fig. 2D) compared to its control in NSCLC H1299 cells.

MMP9 Is the Direct Target of miR-133b

MMP9 was predicted to be a direct target of miR-133b by TargetScan (Fig. 3A). Overexpressing or suppressing miR-133b respectively downregulated or elevated the protein expression of MMP9 in H1299 cells (Fig. 3B), as well as the mRNA expression of MMP9 (Fig. 3C). Luciferase reporter assays were used to determine whether miR-133b could directly target the 3′-UTR of MMP9 in H1299 cells. Cotransfection of miR-133b mimics with MMP9 3′-UTR wt vector significantly decreased luciferase reporter activity ($p = 0.005$) (Fig. 3D, lanes 1 and 2). This effect on luciferase activity was abrogated when cotransfected

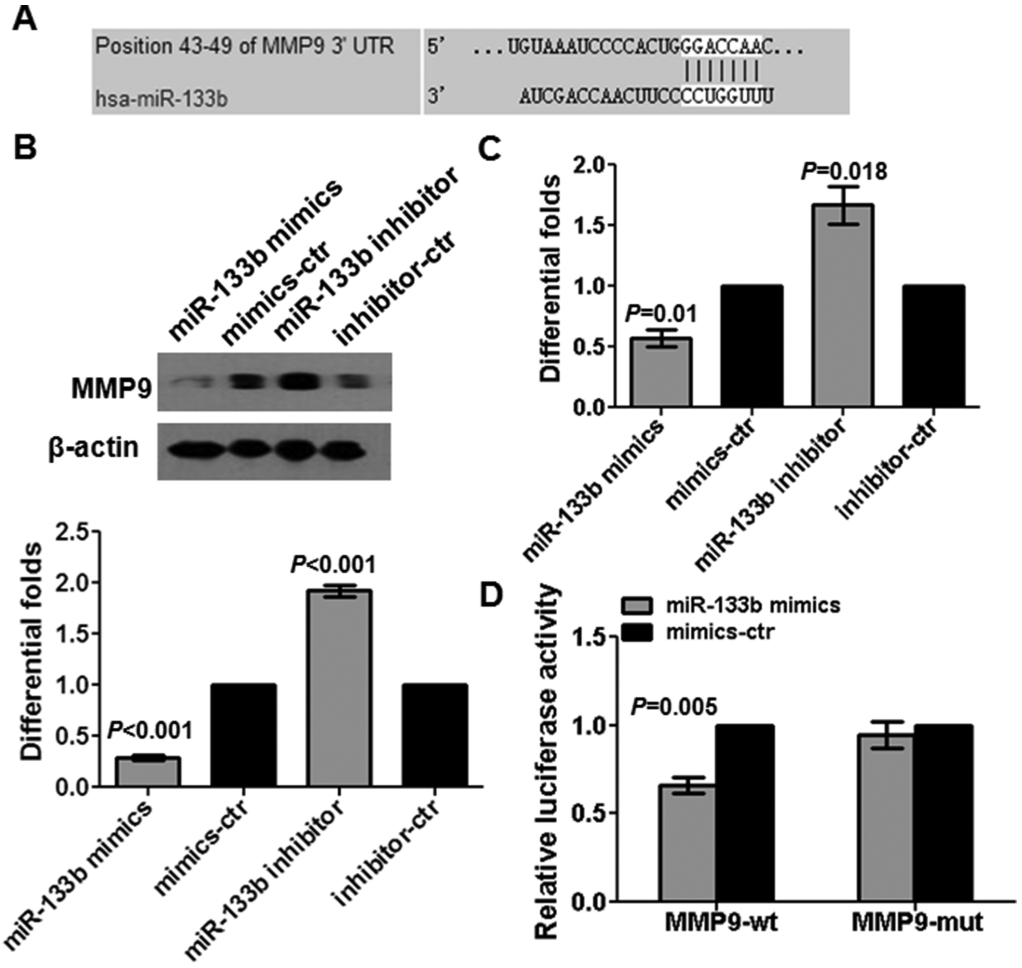

Figure 3. miR-133b directly targeted matrix metallopeptidase 9 (MMP9). (A) Alignment results of miR-133b and MMP9 3′-UTR. (B) MMP9 expression was suppressed or elevated after H1299 cells transfected with 100 nM miR-133b mimics or inhibitor. β-Actin served as the internal control. (C) mRNA levels of MMP9 were reduced or induced after H1299 cells were transfected with 100 nM miR-133b mimics or inhibitor. (D) H1299 cells were cotransfected with miR-133b mimics or negative control and luciferase reporters carrying either the predicted miRNA target site in MMP9 3′-UTR (wt) or its corresponding mutant (mut). Data were presented as mean ± SD for three independent experiments.

with mut vector ($p=0.276$) (Fig. 3D, lanes 3 and 4). Taken together, these results demonstrated that MMP9 was a direct target of miR-133b in H1299 cells.

Suppression of MMP9 Inhibits NSCLC Cell Growth and Invasion

To investigate the role of MMP9 in NSCLC carcinogenesis, siRNA transfection was employed to knock down MMP9 expression in H1299 cells. Knockdown efficiency was evaluated by Western blot (Fig. 4A). Transiently transfecting MMP9 siRNA into H1299 cells not only significantly reduced cell growth (Fig. 4B) but also inhibited cell migration and invasion in H1299 cells (Fig. 4C and D), a role similar to that of miR-133b in H1299 cells.

Correlation Between miR-133b and MMP9 mRNA Levels

We next measured the mRNA levels of miR-133b and MMP9 in NSCLC specimens and their corresponding distal paracarcinoma tissues. The expression level of miR-133b (Fig. 5A) was decreased in NSCLC specimens compared to their corresponding distal paracarcinoma tissues, while MMP9 was significantly increased in cancer tissues (Fig. 5B). As a result, miR-133b expression was negatively correlated with MMP9 expression (Fig. 5C).

DISCUSSION

miR-133b, a muscle-specific miRNA[5,6], has been reported to be deregulated in many kinds of cancer[7–10]. However, its

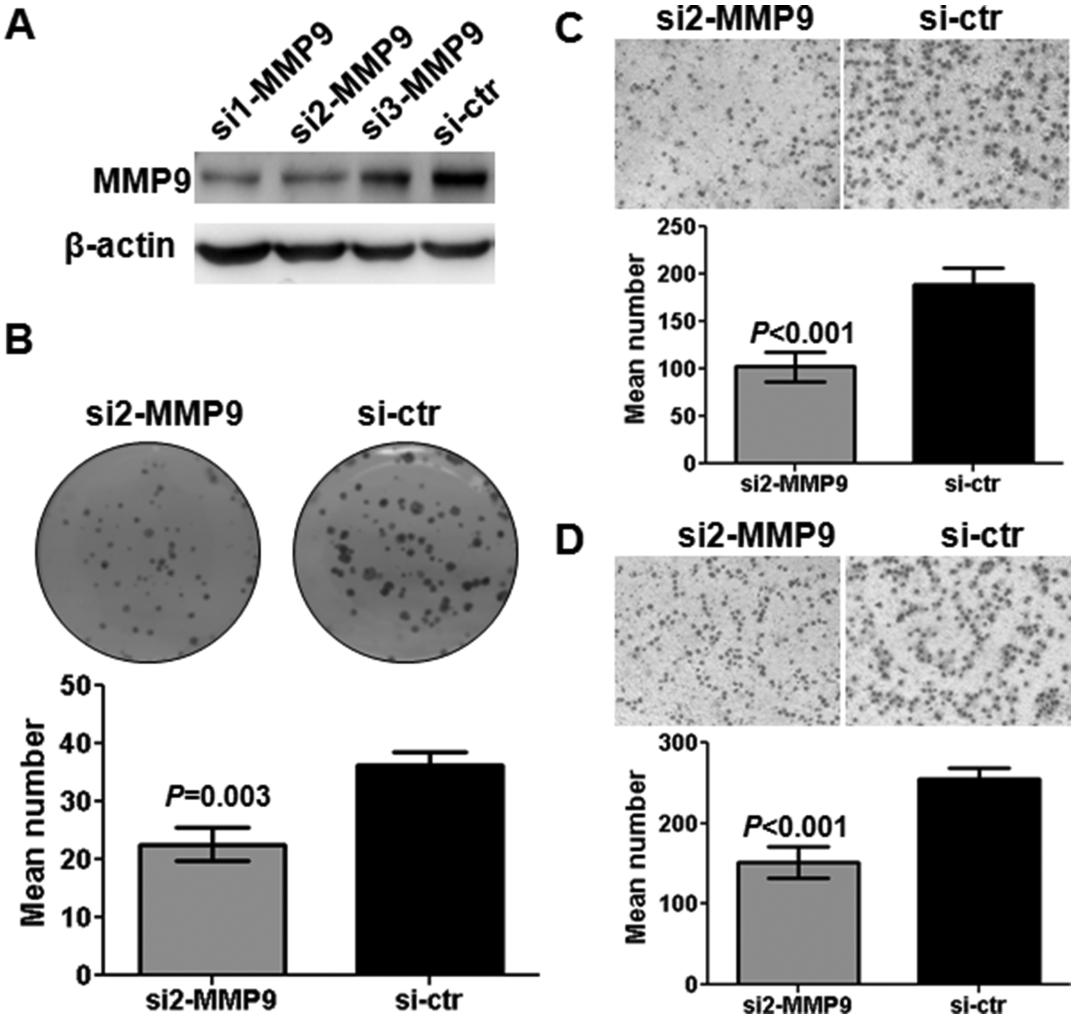

Figure 4. MMP9 knockdown suppressed aggressive phenotypes in NSCLC H1299 cells. (A) Decreased MMP9 protein levels were detected in NSCLC H1299 cells transfected with siRNA by Western blot. (B) Colony formation assay was performed on NSCLC H1299 cells with siRNA2-MMP9 or the control. (C, D) Transiently downregulated MMP9 dramatically inhibited cell migration and invasion in NSCLC H1299 cells. Data were presented as mean ± SD for three independent experiments.

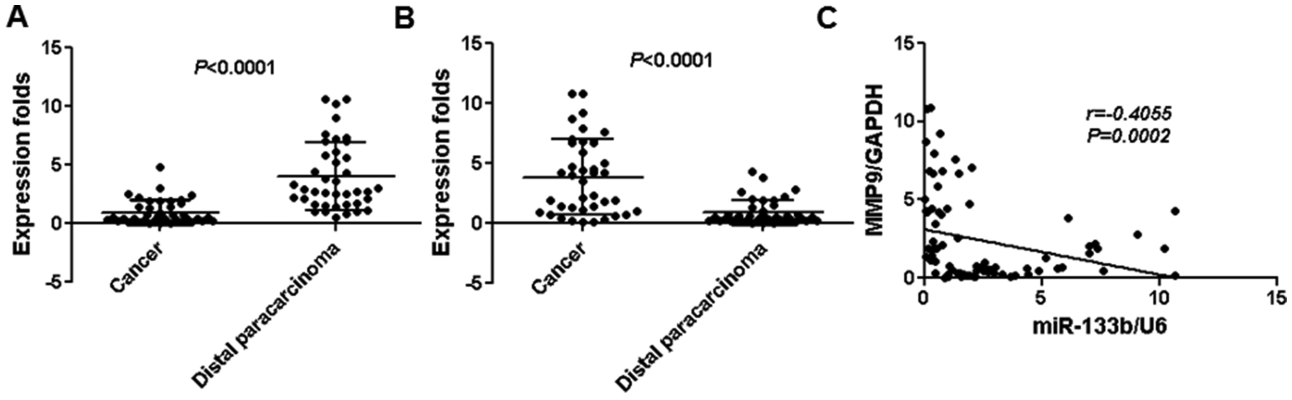

Figure 5. The correlation between miR-133b and MMP9 mRNA levels. (A) miR-133b was markedly decreased in 40 fresh NSCLC tissues compared to their corresponding distal paracarcinoma tissues. (B) MMP9 was significantly upregulated in 40 fresh NSCLC tissues compared to their corresponding distal paracarcinoma tissues. (C) miR-133b expression was negatively correlated with MMP9 expression.

role and molecular mechanism in NSCLC remain largely unknown.

In this study, we observed that miR-133b obviously inhibited cell growth, migration, and invasion in vitro, and the tumor suppressor role of miR-133b in NSCLC was partially through targeting MMP9. These results indicated that miR-133b would be a new diagnostic marker or therapeutic target for NSCLC.

To specifically determine the contributions of miR-133b in the regulation of NSCLC cell phenotypes, transiently increased miR-133b expression in NSCLC cells by miR-133b mimics markedly reduced cell growth, migration, and invasion. On the contrary, transiently knocking down miR-133b expression by miR-133b inhibitors significantly elevated cell growth, migration, and invasion. These results together suggested that miR-133b suppressed cell growth, migration, and invasion in NSCLC. Biological functions of miR-133b found in this study were consistent with previous studies[7,9–11].

Metastasis is a complex, multistep process that involves degradation of the basement membrane and the extracellular matrix[14]. MMP9, secreted by various cancer cells[15–17], is a member of the MMP family, which is involved in the breakdown of extracellular matrix in cancer metastasis[18,19]. Overexpression of MMP9 has been reported to facilitate metastasis of different cancer cells and appears to be an important molecule to promote NSCLC metastasis[20]. In this study, MMP9 3′-UTR was predicted to be an miR-133b-targeted region by bioinformatics assay. Our experiments further validated this speculation, which was consistent with a previous study in renal cell carcinoma[21]. In addition, knocking down MMP9 suppressed cell growth, migration, and invasion, a role similar to that of miR-133b in NSCLC. These results indicated that MMP9 participated in miR-133b-mediated suppression of cell growth, migration, and invasion in H1299 cells.

Consistent with their known roles in NSCLC, we observed a reduced expression of miR-133b but an elevated MMP9 mRNA expression in clinical NSCLC tissues. miR-133b was negatively correlated with MMP9 mRNA expression. Our study underscores the significance of the miR-133b-MMP9 axis in mediating NSCLC pathogenesis.

Taken together, this study provided evidence that miR-133b inhibited cell growth, migration, and invasion through directly targeting MMP9 in NSCLC. Therefore, miR-133b could be a new diagnostic marker or therapeutic target for NSCLC.

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